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TECHNICAL REPORT FOR GRANT #NAG 10-0183/NAG 10-0224

**"Microgravity Effects During Fertilization, Cell Division, Development,
and Calcium Metabolism in Sea Urchins".**

Ground studies and analysis of experiments performed on STS-77

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Introduction and Overview

Calcium loss and muscle atrophy are two of the main metabolic changes experienced by astronauts and crew members during exposure to microgravity in space. For long-term exposure to space it is crucial to understand the underlying mechanisms for altered physiological functions. Fundamental occurrences in cell biology which are likely to depend on gravity include cytoskeletal dynamics, chromatin and centrosome cycling, and ion immobilization. These events can be studied during fertilization and embryogenesis within invertebrate systems. We have chosen the sea urchin system to study the effects of microgravity on cytoskeletal processes and calcium metabolism during fertilization, cell division, development, and embryogenesis. Experiments during an aircraft parabolic flight (KC-135) demonstrated: (1) the viability of sea urchin eggs prior to fertilization, (2) the suitability of our specimen containment system, (3) the feasibility of fertilization in a reduced gravity environment (which was achieved during 25 seconds of reduced gravity under parabolic flight conditions).

Two newly developed pieces of spaceflight hardware made further investigations possible on a spaceflight (STS-77): (1) the Aquatic Research Facility (ARF), and (2) the Fertilization Syringe Unit (FSU). The Canadian Space Agency developed ARF to conduct aquatic spaceflight experiments requiring controlled conditions of temperature, humidity, illumination, and fixation at predetermined time points. It contained a control centrifuge which simulated the 1 g environment of earth during spaceflight. The FSU was developed at the Kennedy Space Center (KSC) by the Bionetics Corporation specifically to enable the crew to perform sea urchin fertilization operations in space. An in-depth description of the design and operation of the ARF and FSU can be found in Schatten et al. (1999b).

Eggs and embryos of either the sea urchin species *Lytechinus pictus* or *Strongylocentrotus purpuratus* were loaded into Standard Container Assemblies (SCAs) which comprised the experimental aquaria (33 mL volume) contained within the ARF. A newly developed Fertilization Syringe Unit (FSU) was used to achieve in flight fertilization capability. Fixative solutions were preloaded into fixation blocks maintained adjacent to the SCAs and injected at pre-selected time points, resulting in final (diluted) concentrations of either 0.5% or 2% glutaraldehyde (depending on the embryonic stage). In-flight fertilization, cell division, and embryo development was successfully achieved on orbit. Light, scanning, and transmission electron microscopy determined that all desired embryonic and cell division stages (16-cell stage, blastula, gastrula, and pluteus) were preserved using the experimental protocols and fixation capability provided by the ARF/FSU system.

Embryos were fixed at time points ranging from 3 hours to 8 days after fertilization. Investigative emphasis was placed upon: (a) sperm-induced calcium-dependent exocytosis and cortical granule secretion, (b) membrane fusion of cortical granules and plasma membranes, (c) microfilament polymerization and microvilli elongation, (d) microtubule and centrosome organization during cell division and cell differentiation, and (e) embryonic development into morula, blastula, gastrula, and pluteus stages. For embryos cultured under microgravity conditions, the processes of cortical granule discharge, fusion of cortical granule membranes with the plasma membrane, elongation of microvilli and elevation of the fertilization coat were reduced in comparison with embryos cultured at 1 g in space and under normal conditions on earth. Also, 4% of all cells undergoing division in microgravity showed abnormalities in the centrosome-centriole complex. These abnormalities were not observed within the 1 g flight and ground control specimens, indicating that certain alterations in sea urchin developmental processes occur under microgravity.

Key Words: Fertilization, Reproduction, Development, Sea Urchins, Spaceflight, Space Biology

Results

This project explores the role of microgravity during fertilization, early development, and cytoskeletal organization. The results obtained in the sea urchin system will provide data that will help us understand how spaceflight affects the cytoskeletal and calcium-sequestering systems that play important roles in bone restructuring and in the muscular and nervous system of the human body. This report is based on (a) ground studies, (b) studies during a parabolic flight on the KC135, and (c) experiments on the space shuttle Endeavor (STS-77).

a) Ground studies (please see also Steffen et al., 1992; Chakrabarti et al., 1995).

Ground studies are described in two of our publications and include analysis of cytoskeletal structures during sea urchin fertilization, cell division, and development. Antibodies to microtubules (Schatten et al., 1992; Schatten 1994) and centrosomes (Schatten, 1994; Thompson-Coffe et al., 1996) were used to determine cell cycle specific changes of cytoskeletal organization during fertilization and cell division, cell differentiation, and embryonic development to the morula, blastula, gastrula, and early pluteus larvae. We have also tested material and prototype culture chambers that would allow fertilization and maintenance of eggs and embryos for long-term culture in space. Fixation and egg storage conditions have been reported in **Chakrabarti et al., (1995)**. Prototype experiments were conducted during an aircraft parabolic flight on the KC135 to test if fertilization would occur under microgravity conditions (**Schatten et al., 1999a**).

b) Studies during a parabolic flight on the KC-135 (please see also Schatten et al., 1999a).

Fertilization Studies: Preflight preparation. Twenty hours prior to KC-135 take-off, *Strongylocentrotus purpuratus* sea urchins were spawned by 0.55 M KCl stimulation. Eggs were shed into 12°C, 0.22 µm Millipore filtered, artificial sea water (ASW) and sperm were collected "dry" on ice. Individual females were checked for eggs yielding the greatest percent fertility and one was selected which gave 98% fertilization. The eggs were then dejellied by five times passage through a 74 µm Nitex mesh and the egg concentration adjusted to 0.15-0.2% (v/v) (approximately 1,000-2,000 eggs/ml). Three hours before KC-135 take-off, additional urchins were spawned and processed as above except that they were stripped of their vitelline layer by a ten minute treatment with 6 mM DL-Dithiothreitol (DTT) and 0.1% trypsin. Two groups of eggs (those with and without an intact vitelline layer) were used so that two different methods could be used to confirm sperm penetration. Eggs with an intact vitelline layer were used to confirm fertilization simply by noting the presence of the fertilization coat whereas eggs stripped of the vitelline layer were unable to form fertilization coats and were labeled with a fluorescent DNA stain (DAPI) to confirm spermatozoa incorporation.

At the beginning of the 0-g period, a diluted sperm solution was injected into Teflon bags which were then manually manipulated. At the end of the 0-g period, a spermicide was added to lyse unincorporated sperm while leaving eggs unharmed. These eggs have the capability of continuing through the cell cycle for the remainder of the KC-135 flight. 2-cell embryos were obtained that had been fertilized during the 0-g portion of a parabola and developed normally through first mitosis. Eggs fertilized in reduced gravity were allowed to develop for the remainder of the KC-135 flight (approximately 1-2 hours) and proceeded through the subsequent cell cycles. Two parameters were important for successful fertilization and development. First, mixing of the gamete solution after sperm injection into the Teflon culture bags by manual manipulation is required for enhanced gamete distribution and interaction, increased fertilization percentages, and synchronous fertilization and embryo development. Second, increased length of time during fertilization in 0-g results in higher rates of fertilization.

c) Experiments on the space shuttle Endeavor (STS-77) (please see Schatten et al., 1999b,c).

ARF Hardware: The Aquatic Research Facility (developed by the Canadian Space Agency) was designed to perform a variety of aquatic experiments within a Space Shuttle mid-deck locker. It provided controlled conditions of temperature and illumination and contained two centrifuges. The microgravity centrifuge rotated once every ten minutes (to avoid position effects during the experiment) and a 1g control centrifuge rotated at 80 RPM, which resulted in an on-orbit 1 g environment based upon centrifugal force. Together, the 1 g and microgravity centrifuges allow the researcher to factor out all non-gravity spaceflight-associated effects (such as radiation, vibrations during lift-off and landings, etc.) and thereby ascertain if the observed results from the spaceflight can be attributed to microgravity conditions.

The culture chambers employed in the ARF system are called Standard Container Units (SCU's) that are subdivided into two Standard Container Assemblies (SCA's; side A and side B) that are each filled with 33ml embryo cultures. Our experiments employed 4 SCU's (8 SCA's) each for microgravity, 1g space, and ground control experiments. The Standard Container Units (SCU's) are referred to as miniaquariums that hold the sea urchin cultures. Each SCA is equipped with a fixative block assembly that is filled with 1.1-1.2mls of a concentrated mixture of fixative that is diluted for fixation of embryos in the specific SCA units. Release of

fixative is actuated by computer control at preset time points to release fixative into the SCA's which will fix the sea urchin embryos at preselected time points.

Samples of sea urchin embryos were fixed for analysis at 180 minutes, 7 hours, 24 hours, and 8 days from space flight, and ground control samples. Postflight analysis with light microscopy determined that fertilization had taken place in the samples fertilized on ground and those fertilized in space. Most stages were obtained with the exception of late-stage plutei. Light microscopy was used to determine that the fertilization coats in the μg and 1g flight controls had not elevated as high as in the ground controls which is an indication that secretion of cortical granules, signal transduction, microfilament activity, and calcium events are affected by microgravity. Similarly, the surface morphology was altered in the late stage embryos cultured in space when compared with those cultured in 1g-space and those cultured on ground. Scanning electron microscopy, transmission electron microscopy, and (immuno)fluorescence microscopy was employed on whole cells and on isolated cytoskeletal structures to analyze microtubules and centrosomes. For the immuno-detection of other proteins and cytoskeletal fibers, cells were sectioned into $1\mu\text{m}$ slices. Our results indicate that secretion, microfilaments and microtubules, as well as phosphorylation and calcium events are altered to a certain extent under microgravity conditions. **Please see also Schatten et al., 1998, Proceedings MSA 56(4)2, 1032-1033 and Schatten et al., 1998, SCANNING Vol. 20,3, p. 223-224.**

1) Fertilization and Cell Division. The overall analysis of embryos cultured in microgravity, 1g in space, and 1g on earth determined that development in the cultures grown in microgravity took place but was delayed when compared with those grown in the 1g centrifuge in space and the cultures grown in the ARF system on earth as well as in beakers kept in our laboratory. This indicates that adaptation processes to the microgravity conditions occur. The percentage of fertilization was determined by the elevation of the fertilization coat and by successful incorporation of the sperm pronucleus which fused with the female pronucleus and allowed successful mitosis and cell division. Normal and abnormal mitoses were determined by immunofluorescence microscopy to analyze centrosomes, microtubules, and DNA. Four percent of dividing cells displayed abnormal mitosis. Although 4% is not a statistically high number, these data are relevant because abnormal centrosomes in single cells can cause abnormal chromosome separation which is associated with genomic instability, a finding that has generated much interest in the field of cancer research. These findings suggest that centrosomes are affected by microgravity.

2) Cell differentiation. The 16-cell stage is most critical for embryo development in the sea urchin system. Differentiation patterns are set up during this early stage which results in the formation of macromeres, mesomeres, and micromeres. The centrosome-centriole complex and microtubules in particular play critical roles in allowing the process of differentiation. This time point confirmed the results obtained for cells at the 2-cell stage which point to 4% abnormalities in the centrosome-centriole complex. Four percent of all developing cells showed abnormalities in cell division and cell differentiation which indicates that embryos in this stage of development are susceptible to effects by microgravity. The centrosome-centriole complex has been shown to be involved in defining polarity in developing systems, and since this complex appears to be affected in microgravity, polarity might be affected by microgravity.

3) Blastula Formation and Cilia Function. We analyzed a) the structure of cilia, b) the centrosome-centriole complex and c) the striated fibers which are associated with cilia in most cell systems. Our results indicate that the striations are not as pronounced in the microgravity-exposed cells but further experiments are needed to determine the significance of this finding. Immunofluorescence and electron microscopy reveals structural alterations in the centriole-centrosome complex. An antibody to intermediate-like filaments, (Ah-6; Schatten et al., 1987) stains the centriole-centrosome complex weaker in microgravity exposed cells. **(Please see also Schatten et al., 1998a,b).**

4) Plutei and Spicule Formation. Contrary to our earlier success culturing cells to the pluteus stages, the growth of embryos to the late pluteus stage of development could not be achieved and analyzed during STS-77 mission because of technical difficulties with the fixation system.

NEWS COVERAGE:

Radio:

- (1) Radio Interview with BBC
- (2) Radio Interview with Wisconsin Public Radio
- (3) Radio Interview with "The Sciences" New York
- (4) Radio Interview with "Let's Watch Radio" - Wisconsin

Newspaper Coverage:

- (1) Wisconsin Weekly
- (2) Wisconsin State Journal
- (3) Wisconsin Capital Times
- (4) Milwaukee Sentinel
- (5) Florida Today
- (6) Other smaller local Florida newspapers

Lectures: A total of 15 lectures were presented

Publications :

1. Steffen, S., Fiser, R., Simerly, C., Schatten, H., and Schatten, G. (1992). Microgravity Effects on Sea Urchin Fertilization and Development. *Adv. Space Res.* Vol 12, No.1 (1)167-(1)173.
2. Chakrabarti A, Stoecker A, Schatten H, 1995. Modification of experimental protocols for the analysis of cytoskeletal structures during fertilization, cell division, and development of sea urchin embryos. *AAAI-95-1059*: p. 1-10.
3. Schatten, H., and Chakrabarti, A. (1996). Culture of sea urchin embryos in the new aquatic research facility (ARF) and first results from its maiden space voyage on STS-77. *Grav. Space Biol., Bull.* 1996, 10(1): 46
4. Schatten, H., Chakrabarti, A., Taylor, M., Crosser, M., and Mitchel, K. (1998). The Centriole-Centrosome Complex is Affected by Microgravity During Cell Division and in Cilia of Sea Urchin Embryos: Results from Space Flight Experiments. *Proceedings MSA 56(4)2*, 1032-1033.
5. Schatten, H., Taylor, M., Chakrabarti, A., Kemp, R., and Crosser, M. (1998). Scanning Electron Microscopy of Sea Urchin Eggs and Embryos Exposed to Microgravity on a Space Shuttle Flight. *SCANNING* Vol. 20,3, p. 223-224.
6. Schatten, H., Chakrabarti, A., and Taylor, M. (1998). The centriole-centrosome complex during cell division and in cilia of sea urchin embryos is affected by microgravity: Results from space flight experiments. *Molecular Biology Abstracts*, University of Missouri-Columbia.
7. Schatten, H., Zoran, S., Levine, H.G., Anderson, K., and Chakrabarti, A. (1999a). Sea urchin fertilization during a KC-135 parabolic flight. *Journal of Gravitational Physiology*, in press.
8. Schatten, H., Chakrabarti, A., Levine, H.G., Anderson, K., Fiser, R., and Taylor, M. (1999b). Utilization of the aquatic research facility and fertilization syringe unit to study sea urchin development in space. *Journal of Gravitational Physiology*, in press.
9. Schatten, H., Chakrabarti, A., Taylor, M., Anderson, K., Levine, H., Runco, M., Kemp, R., and Sommer, L. (1999c). Calcium-dependent secretion, membrane fusion, and cytoskeletal organization is affected by microgravity during fertilization and development of sea urchin eggs and embryos cultured during a space flight. *Molecular Biology Abstracts*, University of Missouri-Columbia.

10. Schatten, H., Chakrabarti, A., Taylor, M., Levine, H., Anderson, K., Runco, M., Kemp, R., and Sommer, L. (1999c). The Effects of Spaceflight Conditions on Fertilization and Embryogenesis in the Sea Urchin *Lytechinus pictus*. Cell Biology International, in press
11. Schatten, H., Chakrabarti, A., Levine, H., Runco, M., Anderson, K., and Taylor, M. (1999). The Effects of Spaceflight On Calcium-Dependent Secretion and Cytoskeletal Organization During Fertilization and Cell Division in Sea Urchin Eggs and Embryos. Proceedings MSA, in press.

PERSONNEL

INVESTIGATOR:

The Principal Investigator on this project, Dr. Heide Schatten, holds a Ph.D. from the University of Heidelberg and the German Cancer Research Center where she studied the effects of anti-cancer on cytoskeletal organization on cancer cell proliferation. Dr. Schatten performed predoctoral and postdoctoral work with Dr. Daniel Mazia at the University of California in Berkeley to study the cytoskeletal and calcium related mechanisms underlying fertilization, cell division, and development, using sea urchin cells as a model for invertebrate and mammalian reproduction. Her professional affiliations include research, teaching, and administrative positions at the Florida State University, the University of Wisconsin-Madison, the University of Illinois at Urbana-Champaign, and the University of Wisconsin-Madison. She has moved to the University of Missouri-Columbia where she holds a position as associate professor and director of the campus-wide Electron Microscopy Core Facility.

RESEARCH ASSOCIATE:

The Research Associate on this project is Dr. Amit Chakrabarti who holds a Ph.D. from the University of Calcutta on plant biology and has been a guest scientist at major research laboratories in Europe to study phosphorylation events in chromosomes and the cytoskeleton of plant and mammalian tissue culture cells. He has been responsible for developing the techniques used for the microgravity studies on sea urchin cells and has been instrumental in defining the parameters that allow culturing sea urchin embryos in the ARF system to study cell and developmental phenomena.

TECHNICAL SUPPORT STAFF AND CONSULTANTS:

Several specialized forms of microscopy have been used in this project. Technical support staff to help with post-flight analysis at the University of Wisconsin-Madison included Ms. Julie Hedrick who has helped perform the analysis of chromosomes, microtubules, and centrosomes with confocal immunofluorescence microscopy and with high resolution electron microscopy that has been performed at the Integrated Microscopy Resource for Biomedical Research. Mr. Randy Massey has provided technical support with conventional electron microscopy performed at the electron microscope facility in the Department of Anatomy. Ms. Renate Bromberg has assisted with analyzing sectioned cell material for simultaneous viewing of electron and light microscopy samples at the Department of Veterinary Medicine. Ms. Heidi Barnhill has assisted with high resolution scanning electron microscopy performed at Russels Lab.

At the University of Missouri-Columbia technical support was provided by Ms. Meghan Taylor, Ms. Raelynn Kemp, and Mr. Mike Crosser. Dr. Hans Ris from the University of Wisconsin, and Dr. David Nishioka at Georgetown University, have consistently provided their expertise for post-flight analysis and the interpretation of data. The Electron Microscopy Core Facility of the University of Missouri-Columbia is acknowledged for help and use of the Hitachi H-600 and JEOL 1200 EX, transmission and the JEOL JSM35 scanning electron microscope.